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The Type VI Secretion system: a versatile bacterial weapon

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Abstract

The Type VI secretion system (T6SS) is protein nanomachine which is widespread in Gram-negative bacteria and used to translocate effector proteins directly into neighbouring cells. It represents a versatile bacterial weapon which can deliver effectors into distinct classes of target cells, playing key roles in inter-bacterial competition and bacterial interactions with eukaryotic cells. This versatility is underpinned by the ability of the T6SS to deliver a vast array of effector proteins, with many distinct activities and modes of interaction with the secretion machinery. Recent work has highlighted the importance and diversity of interactions mediated by T6SSs within polymicrobial communities and offered new molecular insights into effector delivery and action in target cells.

Key Words

Bacterial protein secretion, Type VI secretion system, bacterial effector proteins, polymicrobial communities

Introduction

The Type VI secretion system (T6SS) is a protein nanomachine deployed by many Gram-negative bacteria in order to translocate effector proteins directly into target cells. Following its recognition as the sixth major protein secretion system in Gram-negative bacteria (Mougous *et al.*, 2006; Pukatzki *et al.*, 2006), the T6SS was initially believed to function as a classical virulence factor, namely to deliver effector proteins which destroy or manipulate the cells of eukaryotic host organisms ('anti-eukaryotic T6SS'). However it has subsequently become clear that the primary function of the T6SS is as a device for inter-bacterial competition. In other words, bacteria use the T6SS to deliver toxic anti-bacterial effectors into rival bacterial cells ('anti-bacterial T6SS'). More recently, the range of known uses of the T6SS has expanded further, including action against microbial fungi and scavenging of scarce metal ions. In parallel, recent work has revealed the importance of interactions mediated by anti-bacterial T6SSs in varied polymicrobial communities and offered new molecular insights into effector delivery and action in target cells.

It has been estimated that > 25% of proteobacteria encode at least one T6SS in their genome (Bingle *et al.*, 2008). These T6SSs, whilst sharing the same 13 or 14 core components (TssA-M, PAAR), show considerable variation in terms of gene content and function and can be split into six sub-families (1, 2, 3, 4a, 4b, 5) (Barret *et al.*, 2011; Boyer *et al.*, 2009). Moreover, two further, evolutionarily-divergent, T6SS-like systems have been reported. One is found on the *Francisella* pathogenicity island and designated T6SSⁱⁱ, the other is found in the phylum *Bacteroidetes* and designated T6SSⁱⁱⁱ, whilst the widespread and well-characterised proteobacterial T6SS can be termed T6SSⁱ. T6SSⁱⁱ and T6SSⁱⁱⁱ have distinct components but a common overall mode of action when compared with the canonical T6SSⁱ (Clemens *et al.*, 2015; Russell *et al.*, 2014). A given bacterial species can contain between one and six different T6SSs, with the complement of T6SSs present frequently varying between individual strains. In some bacteria, one T6SS is used for two distinct roles, e.g. the T6SS in *Vibrio cholerae* is both anti-bacterial and anti-eukaryotic (MacIntyre *et al.*, 2010), whilst in other cases different T6SSs fulfil distinct roles, e.g. T6SS-5 and T6SS-1 in *Burkholderia thailandensis* have exclusively anti-host and anti-bacterial activity, respectively (Schwarz *et al.*, 2010). In general, different bacterial species and strains use and tailor their T6SS(s) for specific roles according to the niche and strategy of the organism. In addition to variation in number and type of T6SS itself, there is also considerable diversity in effector portfolio. Furthermore, regulation of T6SS gene expression is highly organism-specific and matched with the biological role the system is required to fulfil. For example, the anti-host T6SS of *B. mallei* is regulated by the global virulence regulator VirAG, whilst the 'defensive' anti-bacterial H1-T6SS of *Pseudomonas aeruginosa* is post-transcriptionally activated by cell damage-derived signals via the RetS/Gac/Rsm pathway (LeRoux *et al.*, 2015; Schell *et al.*,

2007). The aim of this review is to showcase the diversity and breadth of functions mediated by the T6SS and highlight the widespread importance of this system in many contexts.

Mechanism of effector delivery by the T6SS

The T6SS is a large and dynamic ‘nanomachine’ that uses a contraction mechanism to propel an extracellular puncturing structure out of the secreting cell. The force of this propulsion can further drive the puncturing structure, which is decorated with effector proteins, into an immediately-adjacent cell, thus achieving contact-dependent translocation of effector proteins into target cells (summarised in **Figure 1**).

Assembly and firing of the T6SS machinery

As reviewed in detail elsewhere (Brackmann *et al.*, 2017; Cianfanelli *et al.*, 2016b; Clemens *et al.*, 2018; Nguyen *et al.*, 2018), the T6SS is an envelope-spanning apparatus assembled from 14 core components (TssA-M, PAAR) and comprising several distinct sub-assemblies: membrane complex, cytoplasmic baseplate, cytoplasmic contractile sheath and expelled puncturing structure. The puncturing structure consists of a tube of stacked hexameric rings of the Hcp (TssD) protein, topped by a spike made of a VgrG (TssI) trimer with a PAAR domain-containing protein tip. Assembly begins with the formation of the bell-shaped membrane complex, comprising ten or twelve copies each of the outer membrane protein TssJ and the inner membrane proteins TssLM (Durand *et al.*, 2015; Nguyen *et al.*, 2018). The cytoplasmic face of the membrane complex then docks the baseplate, which consists of six TssEF₂G(K₃)₂ ‘wedges’ around a central VgrG₃PAAR unit. TssK acts as a connector linking the baseplate to the membrane complex, by interacting with TssFG and the cytoplasmic domains of TssLM (Cherrak *et al.*, 2018; Nazarov *et al.*, 2018; Nguyen *et al.*, 2018). The Hcp tube can then assemble onto the base of VgrG, extending into the cytoplasm. Simultaneously, a helical sheath structure made of TssBC subunits polymerises around the Hcp tube in an extended, high-energy ‘primed’ conformation (Kudryashev *et al.*, 2015; Renault *et al.*, 2018; Wang *et al.*, 2017). Rapid (< 2 ms) contraction of the TssBC sheath drives the Hcp-VgrG-PAAR structure through the baseplate and membrane complex, out of the secreting cell. Contraction of the TssBC sheath, which can span the width of the producing cell, provides sufficient power and reach for the expelled Hcp-VgrG-PAAR structure to also breach an appropriately located recipient cell (Basler, 2015; Vettiger *et al.*, 2017; Wang *et al.*, 2017). Following contraction, the contracted sheath is specifically depolymerised by the ATPase TssH, whilst the effectors are somehow released inside the target cell. A role for the final core T6SS component, TssA, in capping the distal end of the Hcp-TssBC structure and co-ordinating assembly of the inner tube and the sheath has recently been described (Dix *et al.*, 2018; Zoued *et al.*, 2016).

The contraction-based mechanism of the T6SS is related to the injection mechanism of contractile bacteriophages, with close structural similarities between the Hcp-VgrG-PAAR structure and the phage tail tube and tail spike, between the TssBC sheath and the tail sheath, and between the TssEFG and gp25gp6₂gp7 wedge units (Brackmann *et al.*, 2017; Cherrak *et al.*, 2018). However in contrast with the phage system, the T6SS can be reused for multiple firing events by the same cell. Indeed dynamic cycles of T6SS assembly-contraction-disassembly have been observed using fluorescence microscopy imaging of individual T6SSs in a number of organisms, including *V. cholerae*, enteroaggregative *E. coli* (EAEC), *P. aeruginosa* and *Serratia marcescens* (Basler *et al.*, 2012; Basler *et al.*, 2013; Durand *et al.*, 2015; Ostrowski *et al.*, 2018).

Accessory components

In addition to the core components above, T6SSs are diversified and further tailored for their function by the possession of accessory structural or regulatory components which are present in some, but not all, T6SSs. In some systems an additional membrane complex component, SciZ/TagL, provides a peptidoglycan binding functionality normally present in TssL, whilst, conversely, T6SSs may also co-opt peptidoglycan hydrolase enzymes to assist in the formation of the membrane complex through the cell wall (Aschtgen *et al.*, 2010; Santin & Cascales, 2017; Weber *et al.*, 2016). In EAEC, a membrane-associated TssA-family protein, TagA, interacts with TssA in order to ‘catch’, stop and stabilise the extending TssBC sheath when it reaches the opposite side of the cell (Santin *et al.*, 2018). Although some other T6SSs possess a second TssA-family protein which is likely to function similarly, many do not, leaving open the question of how else this function might be achieved. Another protein, TagJ, may help to recruit TssH to the contracted sheath in some systems (Forster *et al.*, 2014).

Many T6SSs also contain conserved post-translational regulatory components. In *P. aeruginosa* and *S. marcescens*, a membrane-bound protein threonine kinase, PpkA, phosphorylates the T6SS-associated Fha protein, overcoming inhibition mediated by a negative regulator, TagF, and thereby allowing assembly of an active T6SS. An antagonistic phosphatase, PppA, promotes spatial relocation of the T6SS machinery between firing events. Interestingly, the two systems are used to increase the efficiency of T6SS anti-bacterial activity in distinct ways. In *P. aeruginosa*, the TagQRST complex detects incoming T6SS-mediated attacks from neighbouring cells, activating PpkA and thus causing the H1-T6SS to assemble and fire back towards the attacker, making it a ‘defensive’ system. In *S. marcescens*, the upstream regulator of PpkA is a distinct protein, RtkS, and the signal for activation is independent of incoming attack or cell-cell contact, resulting in an ‘offensive’ system which can attack passive or aggressive neighbours (Cianfanelli *et al.*, 2016b; Ostrowski *et al.*, 2018; and

references therein). Other variations also exist, including PpkA-mediated phosphorylation of TssL in *A. tumefaciens* (Lin *et al.*, 2014).

Modes of effector delivery

An important aspect of the versatility of the T6SS is its ability to deliver a large variety of different types of effector proteins. To achieve this, effectors can associate with the expelled Hcp-VgrG-PAAR structure through multiple distinct mechanisms in order to be translocated between cells (**Figure 1**). ‘Cargo’ effectors non-covalently interact with specific Hcp, VgrG or PAAR proteins, whilst ‘specialised’ effectors comprise modular proteins in which additional effector domains are covalently fused to the C-terminus of Hcp, VgrG or PAAR proteins (Cianfanelli *et al.*, 2016b; Durand *et al.*, 2014). Hcp-dependent cargo effectors are relatively small, bind within the lumen of the Hcp hexamer and are recognised and stabilised by this interaction, as first described for the anti-bacterial effectors Tse2, Tse1 (Tae1^{PA}) and Tse3 (Tge1^{PA}) of *P. aeruginosa* (Silverman *et al.*, 2013). VgrG-dependent cargo effectors interact with specific VgrG proteins to sit on the outside of the spike and include many phospholipase effectors, such as Tle1^{EC} from EAEC (Flaughnatti *et al.*, 2016). Examples of PAAR-interacting cargo effectors have also recently been reported, including TseT in *P. aeruginosa* (Burkinshaw *et al.*, 2018). Many different examples of specialised VgrG effectors (also termed ‘evolved VgrGs’) have been described, including VgrG-1 of *V. cholerae*, which possesses a C-terminal actin crosslinking domain (ACD) (Pukatzki *et al.*, 2007). PAAR domain-containing specialised effectors are also widespread and diverse, including many nuclease toxins. They can be based simply on a PAAR domain followed by an effector domain, but also include a group of large polymorphic toxins known as Rhs proteins. In the latter, a conserved central Rhs repeat domain is predicted to form a shell-like structure around a C-terminal effector domain, with different Rhs proteins possessing a multitude of distinct C-terminal domains and associated immunity proteins (Alcoforado Diniz & Coulthurst, 2015; Hachani *et al.*, 2014; Whitney *et al.*, 2014; Zhang *et al.*, 2012). It also appears that effector domains may on occasion be fused at the N-terminal end of PAAR proteins, further emphasizing their modularity (Shneider *et al.*, 2013). A family of specialised Hcp effectors with a number of distinct C-terminal effector domains, present in members of the *Enterobacteriaceae*, has also recently been described (Ma *et al.*, 2017a). Thus all three components of the expelled puncturing structure can be used for both cargo and specialised effector delivery modes. A given T6SS is typically associated with multiple VgrG, PAAR and/or Hcp homologues, with or without specialised effector domains, allowing for delivery of many effectors. Specific combinations of these homologues define functional tube-spike units and determine the effectors translocated by that firing event (Cianfanelli *et al.*, 2016a). However the frequency and significance of the formation of VgrG heterotrimers, or of ‘mixed’ Hcp tubes, is currently unclear.

In some cases, effector recruitment and therefore delivery by the T6SS requires a further ‘chaperone’ or ‘adaptor’ protein. Several unrelated but widespread families of such chaperones have been described to date. EagR/EagT (DUF1795) proteins bind the N-terminal PAAR-containing domains of Rhs and Tse6-like effectors, stabilising transmembrane regions which may ultimately permit the effectors to cross the recipient cell inner membrane and allowing the effector to be loaded onto the cognate VgrG (Cianfanelli *et al.*, 2016a; Quentin *et al.*, 2018). Tap-1/TecL (DUF4213) family proteins allow the interaction and loading of VgrG-dependent cargo effectors, including TseL and Tde1, onto the cognate VgrG. They are modular adaptors, where the C-terminal half of the protein varies with the associated effector, providing a mechanism for new effectors to be horizontally acquired and interact with existing VgrG homologues via recombination within the Tap-1 gene (Ma *et al.*, 2014; Unterweger *et al.*, 2015). A chaperone (TecT) facilitating interaction of a PAAR-dependent cargo effector with the cognate PAAR protein has also been reported, with PAAR competing with a co-chaperone for access to the chaperone (Burkinshaw *et al.*, 2018). None of these families of chaperones appear to be secreted with their effectors, rather their role is to protect and load specific effectors onto the machinery prior to secretion. Consistent with the different routes of effector secretion, there is no universal secretion signal for substrates of the T6SS. No such signal is required for specialised effectors, whilst cargo effectors require specific 3D interactions with their cognate VgrG/PAAR/Hcp protein, assisted in many cases by specific chaperones. One secretion motif (‘MIX’) has been identified in a subset of effectors widespread in *Vibrionaceae* (Salomon *et al.*, 2014); this motif is likely to define an adaptor domain interacting with one of the core components.

Anti-bacterial effectors

T6SSs represent widespread and potent weapons for killing or inhibiting rival bacterial cells, both within and between species. This is achieved by the delivery of broad-spectrum anti-bacterial effectors (**Figure 2**), with a given system typically able to deliver one or more representatives of several different effector families.

Classes and modes of action of T6SS-delivered anti-bacterial effectors

A large number of T6SS effectors which target the peptidoglycan cell wall of recipient bacteria have been identified. These peptidoglycan hydrolases can be divided into at least five families of peptidoglycan amidases (Tae1-4, TaeX), which cleave specific bonds within the peptide cross-bridges of the cell wall, and four families of peptidoglycan glycoside hydrolases (Tge1-3, VgrG-3), which cleave the backbone glycan chains (Brooks *et al.*, 2013; Ma *et al.*, 2018; Russell *et al.*, 2012; Whitney *et al.*, 2013). The inner membrane is also a common target of T6SS effectors. Five families of lipase/phospholipase effector (Tle1-4, includes effectors with phospholipase A₁ or A₂ activity, and

Tle5, phospholipase D) have been described (Flaughnatti *et al.*, 2016; Russell *et al.*, 2013). Additionally two effectors, VasX and Tse4, have been reported to form pores or channels in the membrane (LaCourse *et al.*, 2018; Miyata *et al.*, 2013). All these effectors targeting the cell wall or membrane act in/from the periplasm, being generally non-toxic if expressed in the cytoplasm and in some cases activated by periplasmic insertion of disulphide bonds (Mariano *et al.*, 2018). This suggests that the major destination for incoming effectors is likely to be the periplasm of target cells, which may also inform on why the T6SS does not appear to act against Gram-positive cells which lack this compartment. However T6SSs also deliver effectors which act in the bacterial cytoplasm. A number of T6SS-dependent nuclease effectors have also been described (e.g. DNases Tde1 and RhsAB) with many more, particularly PAAR-containing specialised effectors, predicted to possess DNase, RNase or deaminase activity according to bioinformatic analyses (Koskiniemi *et al.*, 2013; Ma *et al.*, 2017b; Ma *et al.*, 2014; Zhang *et al.*, 2012). T6SS effectors can also act by degrading an essential cytoplasmic cofactor, as revealed by the identification of two families of NAD(P)⁺ hydrolase effectors (Tse6/Tne1 and Tne2) (Tang *et al.*, 2018; Whitney *et al.*, 2015). Recently a T6SS-delivered ADP-ribosyltransferase toxin, Tre1, which modifies FtsZ by the addition of ADP-ribose and thus inhibits cell division, has been described. Such toxins are often used by bacteria against eukaryotic cells but this work suggests they may also be used by several inter-bacterial toxin delivery systems including the T6SS (Ting *et al.*, 2018). Cytoplasmic acting effectors may reach the cytoplasm in several ways: by a minority of T6SS delivery events reaching the cytoplasm directly, by effectors incorporating transmembrane domains to allow their own traversal of the inner membrane, and/or by target cell protein-mediated import similar to Cdi toxins; to date, some evidence has been presented for each possibility (Quentin *et al.*, 2018; Vettiger & Basler, 2016; Whitney *et al.*, 2015; Willett *et al.*, 2015). Whilst numerous and varied T6SS-dependent antibacterial effectors have already been reported, it seems clear that the portfolio of effectors and of effector modes of action will continue to grow, given that many effectors identified to date still have no known or readily-predictable function and that new effectors will be revealed by experimental and bioinformatic analysis of increasing numbers of bacterial strains and species.

Self-protection by specific immunity proteins

Any bacterial cell possessing an anti-bacterial T6SS must possess a means to prevent self-intoxication by its own effectors (cytoplasmic-acting effectors, prior to secretion) and intoxication by effectors delivered into it by its neighbouring sibling cells (all effectors, incoming). This is achieved through specific immunity proteins, which are encoded adjacent to the gene for the cognate effector protein. Immunity proteins reside in the cellular compartment of action of the effector and normally bind tightly to the effector to physically prevent toxicity (**Figure 2**) (Alcoforado Diniz & Coulthurst, 2015).

For example, immunity proteins against peptidoglycan hydrolase effectors are soluble or lipid-anchored periplasmic proteins which specifically bind to their cognate effectors and block the active site (Russell *et al.*, 2011; Srikannathasan *et al.*, 2013; Whitney *et al.*, 2013). Interestingly, the Tri1 immunity protein, which protects against the Tre1 ADP-ribosyltransferase effector, has a novel dual function. In addition to a typical, specific active site occlusion mechanism, it also has an enzymatic ADP-ribosylhydrolase activity which removes the modification added by the effector and confers broad resistance to related toxins (Ting *et al.*, 2018).

Evolution and acquisition of effector-immunity pairs

T6SS-mediated inter-bacterial competition occurs between and within bacterial species, mediated by considerable variation in effector-immunity portfolio, even between strains of the same species. In addition to diversity in the number and type of effector, there is also variation within effector families, resulting in related but specific and mutually-incompatible effector-immunity pairs. In general, effector-immunity pairs appear to be horizontally acquired in an inter-bacterial ‘arms race’. In the case of specialised effectors, there is some evidence, particularly for Rhs proteins, that homologous recombination events allow the facile exchange of one C-terminal effector domain plus cognate downstream immunity gene for another pair, resulting in highly variable loci which can mediate competition between strains (Koskiniemi *et al.*, 2014). For cargo effectors, whilst simple effector-immunity pairs can be acquired, it appears that they are often acquired together with linked chaperone or VgrG proteins which are predicted to allow their delivery in the recipient background (Barret *et al.*, 2011; Fitzsimons *et al.*, 2018; Unterweger *et al.*, 2015). So-called ‘orphan’ immunity proteins, which do not confer resistance to effectors currently possessed by the organism, are frequently encoded downstream of a related ‘active’ effector-immunity pair. They may be retained from formerly-active effector-immunity pairs where the effector has been lost, or represent newly-acquired genes, in both cases likely able to confer protection against effectors delivered by other strains (Alcoforado Diniz *et al.*, 2015; Kirchberger *et al.*, 2017). Many T6SS effectors appear to be modular toxin domains which can be used for inter-bacterial competition in several contexts, such as as cargo or specialised T6SS effectors, effectors delivered by contact-dependent inhibition (Cdi) or Type VII (ESAT/Esx) secretion systems, or toxin domains of colicins. For example, Tne2 domains are found in putative T6SS cargo effectors, in Rhs and smaller PAAR specialised T6SS effectors, and in proteins containing LXG and WXG motifs associated with T7SSs (Tang *et al.*, 2018), whilst CdiA toxin domains are frequently shared with T6SS-associated Rhs proteins and can also be found in putative T6SS cargo effectors (Batot *et al.*, 2017; Poole *et al.*, 2011).

One reason why anti-bacterial T6SSs are so effective is likely to be because multiple distinct effectors can be delivered at the same time. On one hand, this means that a rival cell cannot simply protect itself by becoming spontaneously resistant to one effector or acquiring one immunity protein. Perhaps more importantly, simultaneous delivery of effectors targeting the cell wall, cell membrane and cellular DNA, for example, or attacking one target with several different enzymatic activities, is likely to lead to more efficient killing than any effector alone. Indeed synergy between several different combinations of effectors has been demonstrated in *P. aeruginosa* (LaCourse *et al.*, 2018). This study also suggested that multiple effectors may protect against variations in environmental conditions which might reduce the efficacy of an individual toxin.

Roles of anti-bacterial T6SSs in bacterial communities

The killing activity conferred by anti-bacterial T6SSs can be extremely potent during *in vitro* co-culture experiments, with T6SS-wielding cells often able to virtually eliminate similar numbers of susceptible competitor cells within a few hours. The obvious next question, then, is how that activity is used and relevant in 'real-life' microbial communities and niches. Once T6SS-dependent anti-bacterial activity had been identified (Hood *et al.*, 2010), it became clear that reports of virulence defects in T6SS mutants should be carefully evaluated, since decreased fitness of a T6SS mutant could be due to loss of ability to compete against the resident microbiota or co-infecting pathogens, rather than the T6SS having a direct effect on host cells. Some years on, there is now evidence that anti-bacterial T6SSs can markedly influence the composition of host-associated communities and affect the outcome for the host. Anti-bacterial T6SSs can also play roles in the social behaviour of bacteria, for example recognition of self, and facilitate horizontal gene transfer by releasing DNA from prey cells.

Polybacterial host-associated communities

A number of studies have demonstrated a role for anti-bacterial T6SSs in overcoming colonisation resistance of the gut microbiota towards pathogens. The T6SS of *Salmonella typhimurium* was found to be required for successful establishment of infection in the gut if the resident microbial community was intact, whilst the T6SS of *Shigella sonnei* increased its ability to outcompete commensal *E. coli* and persist in a mouse model (Anderson *et al.*, 2017; Sana *et al.*, 2016). In *V. cholerae*, T6SS-mediated anti-bacterial activity against the host commensal microbiota was shown to increase intestinal colonisation and activate host innate immunity genes in an infant mouse model, and to contribute to colonisation of the middle small intestine in the infant rabbit model (Fu *et al.*, 2018; Zhao *et al.*, 2018). Interestingly, in a *Drosophila* infection model, T6SS-mediated killing of a subpopulation of commensal bacteria was somehow actively required to trigger host destruction by

V. cholerae (Fast *et al.*, 2018). In contrast, in a zebrafish model, removal of a pre-colonised symbiotic species from the gut in a manner dependent on the action of the *V. cholerae* T6SS was not due to anti-bacterial activity, but rather a direct impact of the VgrG-1 ACD on host intestinal movements, emphasizing the potential complexities of T6SS-dependent *in vivo* interactions (Logan *et al.*, 2018). Members of the *Bacteroidales* are major constituents of the human gut microbiota and commonly possess up to three distinct architectures of T6SSⁱⁱⁱ, with T6SS loci frequently transferred among co-resident species in the gut (Coyne *et al.*, 2016). T6SS-mediated competition has been observed between strains of *B. fragilis* *in vivo* and appears to play a key role in establishing a stable community of compatible *Bacteriodes* strains in a given individual (Chatzidaki-Livanis *et al.*, 2016; Verster *et al.*, 2017; Wexler *et al.*, 2016). Anti-bacterial T6SS activity has also been implicated in generating colonisation resistance. A symbiotic *B. fragilis* strain was shown to utilise its T6SS and the effector Bte2 to exclude a pathogen, a toxigenic strain of *B. fragilis*, *in vivo*, protecting the host from colitis (Hecht *et al.*, 2016). It will be interesting to see whether *Bacteriodes* also utilise the T6SS against invading proteobacterial gut pathogens *in vivo*. In the bee gut, competition mediated by anti-bacterial T6SSs, and in particular highly diverse and readily exchangeable Rhs effector domains, appears to be an important driver of fitness and evolution within the microbiota (Steele *et al.*, 2017).

Complex polymicrobial communities are also found associated with plant hosts, in particular within the rhizosphere. Anti-bacterial T6SSs are frequently found in both pathogenic and symbiotic or beneficial plant-associated bacteria (Bernal *et al.*, 2018). This suggests that anti-bacterial T6SSs should be involved in establishing and protecting beneficial plant-associated communities and in invasion of these communities by pathogens. In support of this concept, T6SS-dependent anti-bacterial activity in the plant-protecting rhizosphere bacterium *P. putida* was shown to reduce colonisation and necrosis caused by the phytopathogen *Xanthomonas campestris* when co-infiltrated into *Nicotiana* leaves (Bernal *et al.*, 2017). From the other side, the anti-bacterial T6SS of the plant pathogen *A. tumefaciens* is effective against *P. aeruginosa* within a plant but not on lab media (Ma *et al.*, 2014).

A very specific symbiotic relationship is that between the *Euprymna scolopes* squid and *V. fischeri*, in which the bacteria colonise individual crypts in the squid light organ and ultimately bioluminesce. It turns out that an anti-bacterial T6SS plays a key role in selecting and spatially separating strains of *V. fischeri* colonising the light organ. Individual crypts cannot be colonised by two or more incompatible strains, where incompatibility is conferred by a difference in T6SS and effector-immunity complement (Speare *et al.*, 2018). In general, host-associated bacterial communities typically exist in a biofilm state, where aggregates of cells are adhered to a surface within a polymeric extracellular matrix. This scenario is likely to be conducive to T6SS-dependent interactions, and indeed T6SS

genes are frequently co-regulated with biofilm genes and the T6SS has been shown to allow persistence of *Burkholderia* in a mixed biofilm (Schwarz *et al.*, 2010). On the other hand, it has been suggested that extracellular polysaccharide may, in some cases, provide a physical barrier which reduces the effectiveness of T6SS attacks (Toska *et al.*, 2018).

Social behaviour and acquisition of genetic material

Anti-bacterial T6SSs can represent a means by which one individual strain or genotype can distinguish self from non-self (i.e. from closely related strains). In some cases, this manifests as the formation of a boundary between two populations. In *Proteus mirabilis*, the formation of Dienes lines, macroscopic boundaries between swarms of two non-identical strains, is dependent on T6SS-mediated killing of non-siblings via strain-specific effector-immunity pairs (Alteri *et al.*, 2013; Wenren *et al.*, 2013). Similarly, in *Myxococcus xanthus*, delivery of a T6SS nuclease toxin was shown to be required for the inability of colonies of different strains to merge (Gong *et al.*, 2018). The T6SS has also been proposed to be a means of policing quorum sensing (QS) 'cheats', spontaneous QS mutants which benefit from the production of common goods (e.g. extracellular enzymes) by a population, whilst no longer producing the goods themselves. In *B. thailandensis*, QS activation of effector-immunity gene expression was shown to result in QS mutant cheats being eliminated by QS-proficient cells intoxicating them with effectors they are no longer immune to (Majerczyk *et al.*, 2016). Even within a genetically uniform population, anti-bacterial T6SSs may be able to promote phenotypic homogeneity by allowing fitter cells to eliminate starving or otherwise less-fit cells from the population. Somewhat similar to elimination of QS cheats, starving cells of *M. xanthus* have reduced levels of T6SS and TssI immunity proteins, allowing them to be killed by delivery of the TssE effector by healthy neighbouring cells (Troselj *et al.*, 2018). Mathematical modelling has provided support for the idea that T6SS-mediated inter-bacterial competition can lead to spatial separation of non-identical bacterial populations and suggested that this separation can favour the evolution of co-operation within the segregated population (McNally *et al.*, 2017).

A distinct ecological role for anti-bacterial T6SSs is in facilitating DNA uptake, and thus horizontal gene transfer, in naturally competent bacteria. T6SS-mediated lysis of non-self cells, whether closely or more distantly related, results in release of DNA from the targeted cell which can then be taken up by the T6SS-wielding attacker. This role was first identified in *V. cholerae*, where T6SS gene expression is co-regulated with that of the competence machinery (Borgeaud *et al.*, 2015). Further studies in this organism indicated that T6SS effector-immunity genes themselves can be transferred in this way, thus an attacker can acquire new weapons from its prey (Thomas *et al.*, 2017). Similarly, T6SS-mediated horizontal gene acquisition has been demonstrated in *Acinetobacter* (Cooper *et al.*,

2017; Ringel *et al.*, 2017). Transfer of genetic material from prey *E. coli* to *Acinetobacter* was frequent enough to allow observation of functional transformation in real time, which may help to explain why *Acinetobacter baumannii* is able to acquire antibiotic resistance so rapidly in the clinic (Cooper *et al.*, 2017).

Anti-host T6SS effectors

In addition to widespread and versatile utilisation of anti-bacterial T6SSs, bacteria can also use T6SSs to directly target eukaryotic cells, including those of host organisms. There have been many reports of virulence, host response and host cell interaction phenotypes dependent on a functional T6SS, in a range of bacterial pathogens (Hachani *et al.*, 2016). However given the caveat that such phenotypes may be an indirect result of the action of an anti-bacterial T6SS, for example against the host microbiota, it is pertinent to consider only those cases where T6SS effectors responsible for direct action against host cells have been identified. During the first decade of T6SS research, these were few in number: VgrG proteins with C-terminal actin crosslinking, actin ADP ribosylase and host membrane fusion domains; another VgrG protein with a tubulin binding domain which modulates microtubule-mediated bacterial internalisation; and two cargo phospholipase D effectors which also facilitate internalisation by binding Akt and activating the PI3K pathway; all reviewed by Hachani and coworkers (Hachani *et al.*, 2016). These phospholipase effectors, PldA and PldB of *P. aeruginosa*, are also anti-bacterial effectors (Tle5 family) which exert toxicity from the periplasm of target cells lacking the cognate immunity proteins. This highlights another aspect of the versatility of the T6SS: not only is the same system sometimes able to deliver dedicated anti-eukaryotic and anti-bacterial effectors (e.g. the ACD-containing VgrG-1 and peptidoglycan hydrolase-containing VgrG-3 proteins delivered by the *V. cholerae* T6SS), but some effectors are able to act trans-kingdom, against both eukaryotic and prokaryotic cells (Brooks *et al.*, 2013; Jiang *et al.*, 2014).

More recently, a number of other anti-host effectors have been reported. The T6SS of *Francisella tularensis* is required for virulence and intracellular proliferation, via phagosomal escape into the cytoplasm. Four effectors, PdpCD and OpiAB, delivered by this system and contributing to intramacrophage growth have been identified. PdpC is the major determinant of phagosomal escape, whilst OpiA can assist in this process through its PI(3)-kinase activity (Brodmann *et al.*, 2017; Eshraghi *et al.*, 2016; Ledvina *et al.*, 2018). One of the first T6SS effectors identified, EvpP of *Edwardsiella tarda*, was recently shown to prevent activation of the NLRP3 inflammasome by inhibiting the Ca²⁺-dependent MAPK-Jnk pathway, whilst TecA of *B. cenocepacia* was shown to cause activation of the Pyrin inflammasome via its Rho GTPase deaminase activity and the resulting cytoskeleton disruption (**Figure 3**) (Aubert *et al.*, 2016; Chen *et al.*, 2017). Actin rearrangement is

also induced by a CNF1-like toxin delivered by *V. parahaemolyticus*, whilst a Tle4-family phospholipase of *P. aeruginosa* causes disruption of the endoplasmic reticulum (Jiang *et al.*, 2016; Ray *et al.*, 2017). In addition, a catalase effector from EHEC, KatN, was proposed to act against reactive oxygen species within host cells (Wan *et al.*, 2017).

The T6SS as a weapon against eukaryotic microbial competitors

It is clear from the studies described in the previous section that the bacterial T6SS is able to deploy effector proteins against eukaryotic cells, and it is also well-appreciated that many polymicrobial communities, including those relevant clinically, contain both bacteria and fungi (Peleg *et al.*, 2010). Therefore it is perhaps not surprising that the versatility of the T6SS extends to its use against fungal cells. Early indications that this could be the case came from observations of T6SS-dependent inhibition of the yeast *Cryptococcus carnescens* by the phytopathogen *P. syringae* and an increase in T6SS gene expression in a biocontrol strain of *P. fluorescens* when colonising plant roots in the presence of the fungal phytopathogen *Gaeumannomyces graminis* (Haapalainen *et al.*, 2012; Marchi *et al.*, 2013). Recently, the T6SS of *S. marcescens*, previously believed to be exclusively an anti-bacterial T6SS, was shown to possess anti-fungal activity against *Sacchomyces cerevisiae* and *Candida* spp., and it was by studying this system that the first T6SS-delivered anti-fungal effector proteins were identified. These effectors, Tfe1 and Tfe2, have distinct actions against target fungal cells, ultimately leading to fungal cell death. Tfe1-mediated intoxication leads to plasma membrane depolarisation without the formation of aspecific pores, whilst Tfe2 intoxication disrupts nutrient uptake and amino acid metabolism and leads to the induction of autophagy, probably as a starvation response (**Figure 3**) (Trunk *et al.*, 2018). Tfe1 and Tfe2 represent new classes of effector proteins, with no obvious similarity to other effectors or proteins of known function, and their precise mode of action remains to be elucidated. It is likely that T6SS-dependent anti-fungal activity is widespread. Not only can homologues of Tfe1 and Tfe2 be detected in other T6SS-wielding bacteria, but typical T6SS effector identification criteria are likely to miss anti-fungal effectors. These criteria include (1) association with T6SS genes, (2) anti-bacterial activity and adjacently-encoded immunity protein, and/or (3) the presence of known toxin or effector domains, all of which would have excluded Tfe1 and Tfe2. This highlights the importance of unbiased approaches such as secretomics for the identification of new effectors. The discovery of T6SS-dependent anti-fungal activity suggests that the contribution of the T6SS to shaping polymicrobial communities is broader and more important than previously appreciated. Extending this idea further, there is also evidence that the T6SS can also act against other single-celled eukaryotes, namely amoebae. Indeed the T6SS was first identified through the requirement for an intact T6SS for *V. cholerae* to resist predation by *Dictyostelium*

discoideum (Pukatzki et al., 2006). Further work revealed that the pore-forming effector VasX and the actin crosslinking effector VgrG-1 are required for virulence against *Dictyostelium*, in addition to having anti-bacterial activity or anti-host activity, respectively (Miyata et al., 2011; Zheng et al., 2011). Thus the *V. cholerae* T6SS can be utilised against bacterial competitors in the host or environment, against amoebal predators in the environment, and against the host directly, representing a truly versatile and multi-purpose weapon.

A contact-independent role for T6SS in metal uptake

A further role for the T6SS, this time not requiring effector delivery into target cells, has also been described. In this case, the T6SS is responsible for secretion of effectors to the extracellular milieu which allow the bacteria to take up particular metal ions. In *Yersinia pseudotuberculosis*, T6SS-4 secretes a Zn²⁺-binding protein, YezP, under conditions of oxidative stress. Secreted YezP is proposed to scavenge Zn²⁺ ions and thus contribute to survival in the presence of reactive oxygen species produced by the host (Wang et al., 2015). Similarly, *B. thailandensis* T6SS-4 secretes a related Zn²⁺-binding effector, TseZ, which allows Zn²⁺ uptake under conditions of oxidative stress via interaction of extracellular TseZ with the TonB-dependent outer membrane haem receptor HmuR (Si et al., 2017a). The *B. thailandensis* T6SS-4 is also responsible for the secretion of another metallophore effector, TseM. TseM is required for manganese uptake under conditions of oxidative stress and interacts with MnoT, another TonB-dependent outer membrane receptor, to achieve transport of Mn²⁺ into the cell (**Figure 3**) (Si et al., 2017b). Importantly, it was demonstrated that possession of TseZ/HmuR or TseM/MnoT, and T6SS-4, by *B. thailandensis* provides a competitive advantage against other bacteria during co-culture in conditions where the respective metal ion is limiting but cells are not in contact. Thus T6SS-dependent secretion of metallophores to the media allows the producing organism to effectively scavenge scarce metal ions from the environment and thus outcompete bacterial competitors without directly harming them. Both metal uptake systems were also required for full virulence in a *Galleria* model, indicating an important role in metal acquisition within a host environment (Si et al., 2017a; Si et al., 2017b). It is not yet clear whether T6SS-4 in *B. thailandensis* and *Y. pseudotuberculosis*, and related systems in other organisms, are also used to deliver toxic effector proteins into target cells, or whether they are used exclusively for metallophore secretion; certainly their transcriptional regulation, responsive to oxidative stress and metal limitation, appears to be tailored for a metal scavenging function.

A distinct role for a T6SS effector in metal uptake has been reported for the *P. aeruginosa* H3-T6SS-dependent effector TseF. Extracellular TseF binds PQS-Fe²⁺ complexes incorporated within outer membrane vesicles (OMVs). (PQS, Pseudomonas Quinolone Signal, is a quorum sensing signalling

molecule with iron chelating properties.) TseF also interacts with the outer membrane siderophore receptor FptA and porin OprF and is proposed to mediate Fe²⁺ uptake by delivering OMV-associated PQS-Fe²⁺ to these receptors for transport into the cell (Lin *et al.*, 2017). It is currently unclear why TseF or the metallophores above would be secreted by the T6SS, a system normally considered to be designed for effector translocation into cells. Nevertheless, these effectors once again highlight the breadth and versatility of T6SS function, as well as raising the possibility that other proteins that function extracellularly may utilise the T6SS for their secretion.

Concluding Remarks

It is now clear that the bacterial T6SS is used for an impressively broad range of functions, all linked by the purpose of increasing the competitive fitness of the secreting cell. While anti-host T6SSs allow the bacterium to compete with host defence mechanisms, anti-bacterial and anti-fungal T6SSs allow the bacterium to compete with rival microbes, both closely and distantly related. Furthermore, those T6SSs and effectors used for contact-independent metal scavenging allow the secreting bacterium to compete with both host defences and co-resident microbes. This functional versatility is permitted by an ever-growing repertoire of diverse effector proteins, and the ability to hook them up to the delivery device in many different ways. It is likely that the portfolio of known effector proteins will continue to increase rapidly, particularly given the evolutionary pressures of inter-bacterial competition and the recent realisation that effectors may also have non-toxic extracellular roles. The extent of T6SS-related contributions to myriad bacterial interactions has further expanded by the validated inclusion of T6SSⁱⁱ (*Francisella*) and T6SSⁱⁱⁱ (*Bacterioidetes*) alongside the canonical T6SSⁱ systems, with an even more distantly-related T6SS-like system ('T6SS^{iv}') recently described (Bock *et al.*, 2017). Importantly, recent work has not only revealed new molecular and atomic-level details of the mechanism of this intriguing machinery, but it has also begun to demonstrate the importance of T6SS-mediated cell-cell interactions in a variety of 'real-life', or at least relevant model, communities. It is likely that the future will reveal many more examples of communities whose composition, dynamics and properties will be shaped by T6SSs of several flavours. Finally, it is exciting to note that T6SS could provide several potential opportunities for the development of new antimicrobial strategies. It might be possible to inhibit the T6SS itself to reduce the fitness of a T6SS-wielding pathogen, although the broad distribution of the system in other bacteria including commensals would require consideration of specificity. Another avenue, although with similar issues when considering complex communities, could be generating engineered 'biocontrol' strains to target pathogens of concern. Finally, by studying T6SS effectors and the impact that they have on target cells, we may learn more about the basic physiology of host, bacterial or fungal cells and how

485 to effectively inhibit them. From both a fundamental and translational point of view, exciting times
486 lie ahead regarding this versatile, widespread and effective bacterial nanoweapon.

487 **Author statement**

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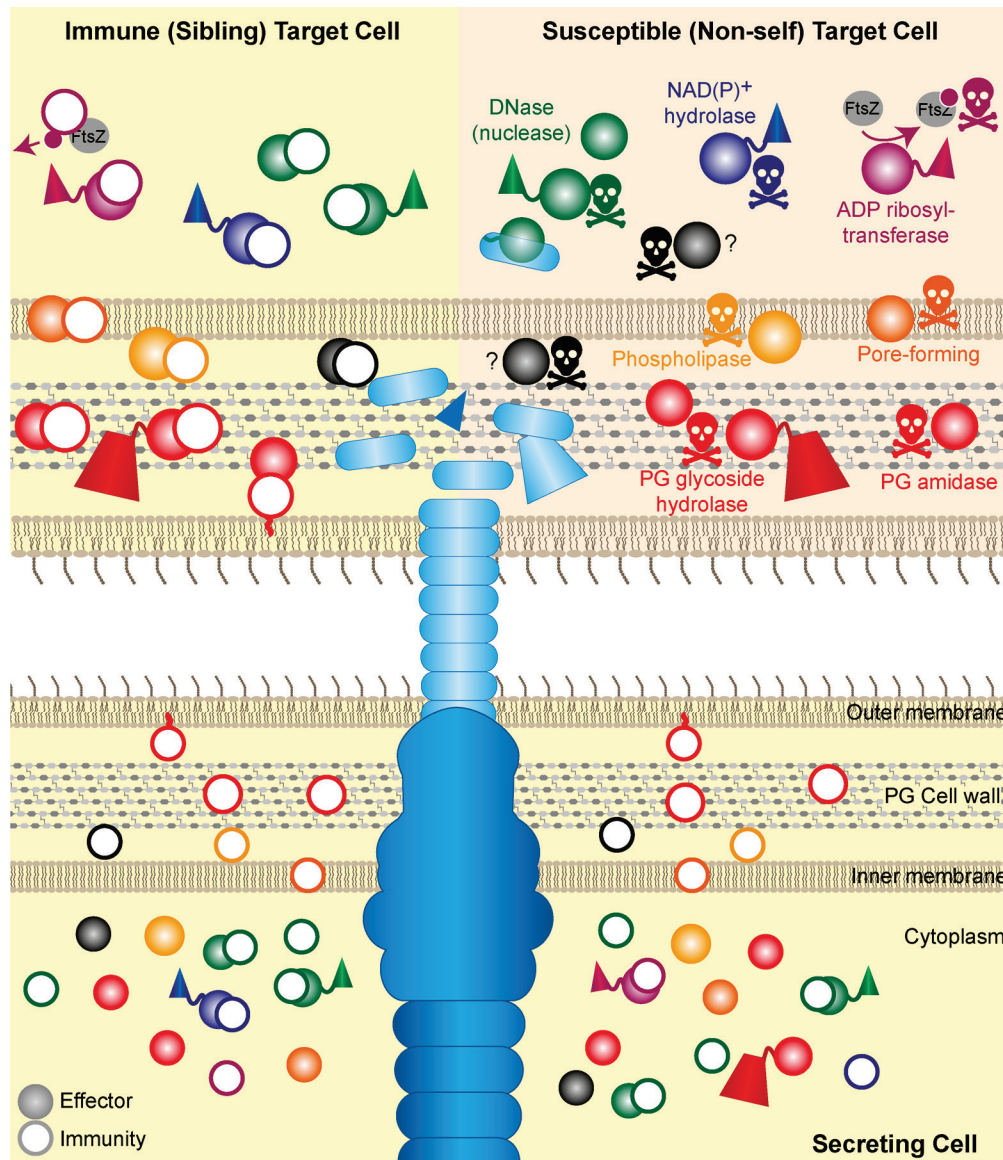


Figure 2. Anti-bacterial effectors and cognate immunity proteins.

Schematic illustration of modes of action of T6SS-delivered anti-bacterial effector proteins and their neutralisation by self-protecting immunity proteins. The T6SS can deliver a variety of toxic effector proteins into a target cell, which may act on different cellular targets including the peptidoglycan cell wall, cellular nucleic acids and the inner membrane. Immunity proteins specific for each individual effector are localised at the site of action of their cognate effector and typically neutralise the toxin by direct binding and physical inhibition. Immunity proteins protect the secreting cell from its own toxins prior to secretion, if they act in the cytoplasm and are not shielded by another structure such as an Rhs repeat domain (this possibility is not depicted here). Immunity proteins also protect genetically identical cells from the action of all the effectors delivered by their neighbouring sibling. Note that only one PAAR-containing protein and one VgrG trimer can be delivered in a single firing event, although several are included here for illustrative purposes. PG, peptidoglycan.

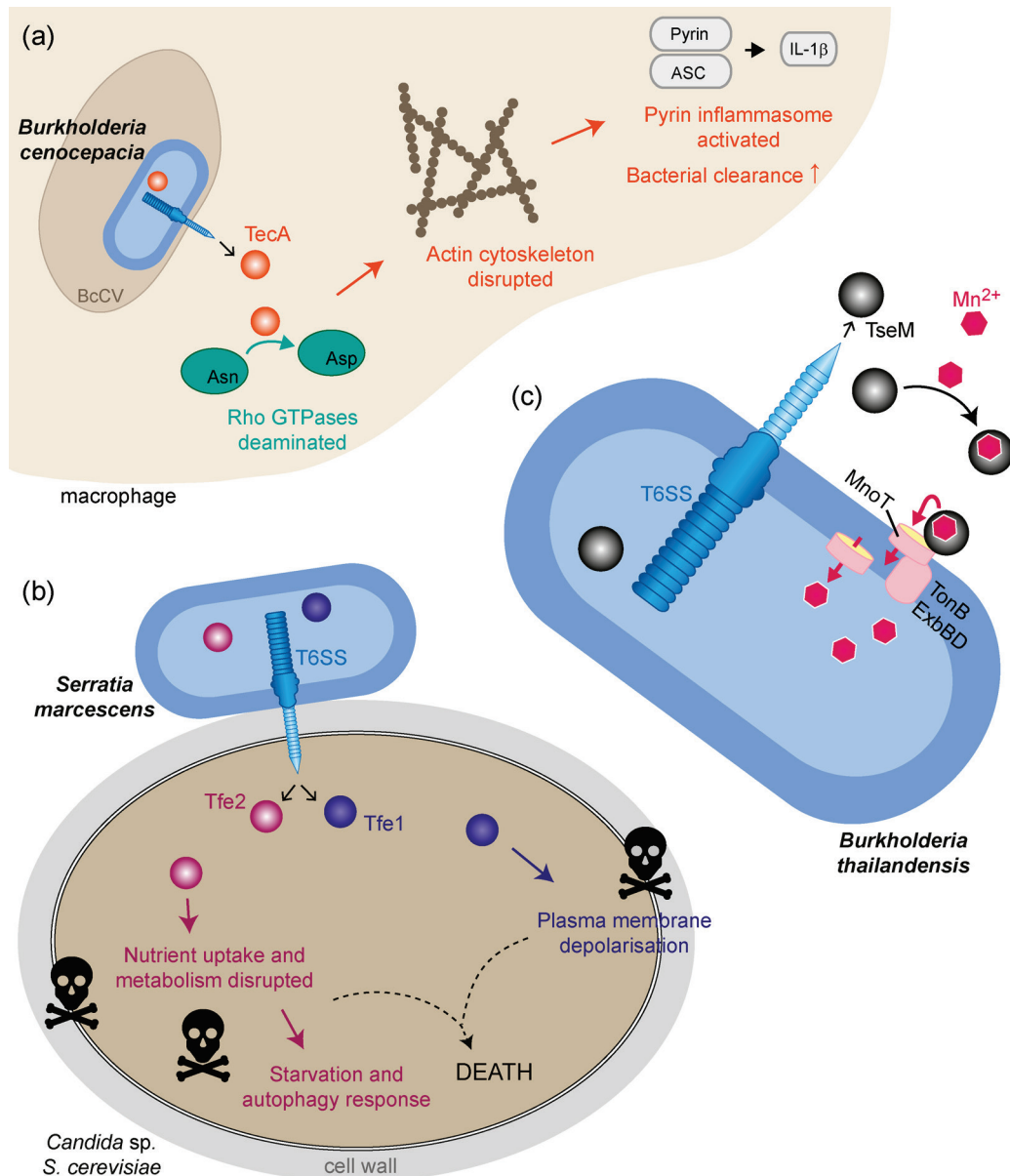


Figure 3. Examples of Type VI secreted effectors with roles distinct from anti-bacterial toxins.

Schematic illustration of current models for the action of the anti-host effector TecA (a), anti-fungal effectors Tfe1 and Tfe2 (b), and metallophore effector TseM (c). (a) *B. cenocepacia* delivers TecA from within the *B. cenocepacia* containing vacuole (BcCV), causing deamidation of specific asparagine residues in Rho family GTPases RhoA and Rac1, leading to disruption of the actin cytoskeleton, activation of the pyrin inflammasome and pyroptosis and increasing bacterial clearance (Aubert *et al.*, 2016). (b) *S. marcescens* delivers Tfe1 and Tfe2 into fungal cells, including *C. albicans* and *S. cerevisiae*. The action of Tfe1 leads to depolarisation of the fungal plasma membrane without formation of large aspecific pores. The action of Tfe2 disrupts inter-related pathways involved in sulfate assimilation, plasma membrane nutrient transport and amino acid metabolism, leading to a starvation response including induction of autophagy. Intoxication by Tfe1 and Tfe2 can eventually cause cell death (Trunk *et al.*, 2018). (c) *B. thailandensis* uses its T6SS-4 to translocate TseM to the extracellular milieu, where it binds Mn²⁺. TseM loaded with Mn²⁺ interacts with the outer membrane TonB-dependent receptor MnoT, which is associated with a TonB-ExbD-ExbB complex, transferring Mn²⁺ from TseM to MnoT and allowing its active import across the outer membrane. Either the SitABCD or MntH transporters may then be utilised to import Mn²⁺ across the inner membrane (Si *et al.*, 2017b).